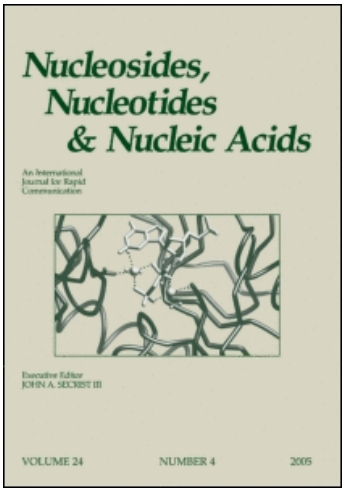

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AN ASSAY SYSTEM TO DETECT THE SELECTIVE ADVANTAGE OF ANTI-HIV RIBOZYME EXPRESSING CD4$^+$ T-LYMPHOCYTES

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ABSTRACT

This study was undertaken to establish an assay system to detect the survival advantage of anti-HIV ribozyme expressing cells under the selective pressure of HIV infection. In a mixture with wild type cells the proportion of ribozyme expressing cells was increased up to 12-fold. As a mechanism of the selective advantage an inhibition of HIV induced apoptotic cell death could be demonstrated. Furthermore, a dose dependency of the antiviral ribozyme effects was observed.

INTRODUCTION

Anti-HIV ribozymes have been demonstrated to be efficient inhibitors of HIV-1 replication *in vitro* (1–4). Thus, clinical studies with retrovirally expressed anti-HIV ribozymes have been initiated (5). In addition to a sufficient inhibition of HIV replication, a selective advantage of cells expressing anti-HIV ribozymes would be a prerequisite for the success of a ribozyme based gene therapy of HIV infection. Especially, the expression of anti-HIV ribozymes in CD4$^+$ T-cells can only be effective if these cells possess an advantage in comparison to unprotected

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cells. However, there have been no *in vitro* or *in vivo* studies demonstrating a selective advantage of ribozyme expressing cells. This study was undertaken to prove whether such a selective advantage exists. To establish an assay to detect the selective advantage a retroviral vector coding for a hairpin ribozyme directed against the 5′LTR of HIV and for the low affinity nerve growth factor receptor (LNGF-R) was designed (2,6).

MATERIAL AND METHODS

Ribozyme and vector design. The ribozyme sequence was expressed by a LN based retroviral vector. The neomycin resistance gene was replaced by the LNGF-R (intracellularly truncated low affinity nerve growth factor receptor) gene. The ribozyme sequence was inserted in the vector upstream of the LNGF-R gene. The vector containing the ribozyme sequence was defined as LHPLtr. In addition to LHPLtr a control vector coding for an inactive ribozyme (HPLtrIA) targeted against the same target sequence as LHPLtr was designed. In this control vector a AAA triplet in the second loop of the hairpin ribozyme was replaced by CGT (2). The structure of the vectors is depicted in Figure 1. The cDNA of the ribozyme and the control sequence were synthesized as two complementary synthetic oligonucleotides (Interactiva, Ulm, Germany). The oligonucleotide sequences were as follows:

HPLtr A, 5′- GGC CGC ACT AGT ACA CAA CAA GAA GGC AAC CAG AGA AAC ACA CGT TGT GGT ATA TTA CCT GGT AC-3′; HPLtr B, 5′- TCG AGT ACC AGG TAA TAT ACC ACA ACG TGT GTT TCT CTG GTT GCC TTC TTG TTG TGT ACT AGT GC -3′; HPLtrIA A, 5′- GGC CGC ACT AGT ACA CAA CAA GAA GGC AAC CAG AGC GTC ACA CGT TGT GGT ATA TTA CCT GGT AC -3′; HPLtrIA B, 5′- TCG AGT ACC AGG TAA TAT ACC ACA ACG TGT GAC GCT CTG GTT GCC TTC TTG TTG TGT ACT AGT GC -3′.

Production of retroviral vectors. The retroviral vectors were transiently transfected into the amphotropic Phoenix packaging cell line (kindly provided by G. Nolan, Stanford University, Stanford, CA) by the calcium-phosphate method (7). High titer virus supernatants were produced by the packaging cell line FlyRD18 (8). 20 μg of plasmid DNA and 2 μg of the plasmid pSV40neo expressing the neomycin resistance gene were co-transfected into FlyRD18 cells by calcium-phosphate precipitation. To generate stable packaging cell lines FlyRD18 cells were selected for neomycin resistance for 2 weeks at 37°C and 5% CO_2 in DMEM supplemented

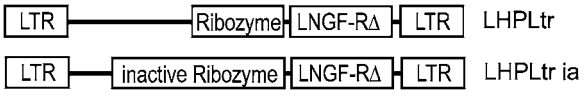

Figure 1. Structure of the retroviral vectors. LHPLtr codes for a hairpin ribozyme (HPLtr) directed against the second GUC triplet in the LTR of HIV-1 HXB2. LHPLtrIA codes for an inactive ribozyme (HPLtrIA) against the same target sequence.

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with 1mM sodium pyruvate, 100 units/ml penicillin, 100 μg/ml streptomycin, 5% sodium bicarbonate, 10% heat inactivated fetal calf serum (Serva, Heidelberg, Germany), and 1 mg/ml G418 (Gibco, Karlsruhe, Germany).

Cell isolation and culture. Peripheral blood mononuclear cells from healthy adult volunteers were isolated by Ficoll density gradient centrifugation. Primary $CD4^+$ T-cells were purified by immunomagnetic cell sorting using a MACS cell sorting device (Miltenyi Biotec, Bergisch Gladbach, Germany). $CD4^+$ T-cells were cultured in RPMI-1640 (GIBCO) supplemented with 100 units/ml penicillin, 100 μg/ml streptomycin, 10 μM Hepes, 10% heat inactivated FCS (Serva), 200 units/ml IL-2 (Genzyme, Rüsselsheim, Germany), 100 ng/ml OKT3 (Ortho, Neckarsulm, Germany), and 10 ng/ml IL-1β (Genzyme) at 37°C. Hut78 cells were cultured in RPMI-1640 supplemented with 100 units/ml penicillin, 100 μg/ml streptomycin, 0.1 mM sodium pyruvate, and 15% heat inactivated FCS (Serva, Heidelberg, Germany).

Transduction. For transduction Hut78 cells were suspended in Phoenix culture supernatant supplemented with 4 μg/ml protamin sulfate (Gibco) in 12 ml tissue culture tubes (Greiner, Nürtingen, Germany). After a 90 min. 2500 rpm spinoculation at 32°C cells were incubated for another 2.5 h at 37°C in 5% CO_2. Then supernatants were removed and the cells were cultured as described above. Prior to transduction $CD4^+$ T-cells were pre-cultured as described above for 48 h. For transduction 5×10^5 $CD4^+$ T-cells were suspended in 3 ml FlyRD-18 culture supernatant supplemented with protamin sulfate, IL-2, OKT3, and IL-1β in 12 ml tissue culture tubes. The transduction was performed as described for Hut78 cells. 24 h after the first spinoculation the transduction was repeated.

Analysis and purification of transduced cells. LNGF-R expression was analyzed by flow cytometry on a FACScan. Cells were stained with anti-LNGF-R mAb (Boehringer Mannheim, Germany). Transduced Hut78 cells expressing LNGF-R were isolated by immunomagnetic cell sorting using a MACS cell sorting device (Miltenyi Biotec) or by fluorescence activated cell sorting on a FACSCalibur (Becton Dickinson). In addition to the whole LNGF-R^+ fraction cells were sorted with respect to the density of LNGF-R expression as high (HE, FL-1 channel >300), medium (ME, FL-1 channel 80–300) and low (LE, FL-1 channel 30–80) LNGF-R expressing cells.

HIV-1 infection. Hut78 and primary $CD4^+$ T-cells were infected with virus supernatants derived from chronically HIV-1 IIIb infected H9 cells. Cells were infected with a MOI of 0.01. Every other day, half of the cell suspension was removed and replaced by fresh culture media supplemented as described above. Weekly the number of viable cells was determined by trypan blue exclusion and culture supernatants were collected. HIV-replication was monitored by determination of the p24 concentration (p24 ELISA, Coulter, Krefeld, Germany) in the culture supernatants.

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Selective advantage. To determine the selective advantage after exposure to HIV, $CD4^+$ T-cells transduced with LHPLtr or LHPLtrIA were mixed with unmodified cells. The rate of LNGF-R positive cells was adjusted at 2–6%. These cell mixtures were then infected with HIV. 7, 14, 21 and 28 days after HIV-1 challenge cell aliquots were harvested and analyzed for the percentage and density of LNGF-R expression by FACS.At different density levels of LNGF-R expression (Hut78: all $LNGF\text{-}R^+$ cells, the upper half [Fl-1 channel >180] and high LNGF-R expression [Fl-1 channel >300]; primary $CD4^+$ T-cells: all $LNGF\text{-}R^+$ cells and high LNGF-R expression [Fl-1 channel >200]) the percentage of LNGF-R positive cells with a LNGF-R expression which was stronger than this thresholds was determined. To quantify the selective advantage at different density levels of LNGF-R expression a ratio of the percentage of LNGF-R positive cells after HIV infection and without HIV inoculation was defined as the relative selective advantage (RSA). To elucidate the mechanism of a selective advantage the percentage of apoptotic cells was determined by flow cytometry after a 20 min. pre-incubation with 7-amino actinomycin D (7-AAD, 20 μg/ml) as described by Schmid et al. (9). The rate of apoptotic cells was determined for all LNGF-R and for highly LNGF-R positive cells.

RESULTS AND DISCUSSION

The assay to quantify an advantage for anti-HIV ribozyme expressing cells based on the principle that the selective pressure of HIV should give the ribozyme expressing cells a selective advantage when competing with wild type cells. To discriminate between ribozyme expressing and wild type cells by flow cytometry, a retroviral vector coding for the ribozyme and for the LNGF-R as a reporter was designed. Since, we showed by RT-PCR that the amount of ribozyme transcripts is strictly correlated with the level of LNGF-R protein expression, the vector allowed us to easily detect flow cytometrically ribozyme expressing cells as well as the level of ribozyme expression. In a mixture of wild type with ribozyme expressing cells a selective advantage for ribozyme expressing cells should result after a challenge with HIV in an increasing percentage of LNGF-R positive cells. To quantify the selective advantage, a ratio designated as relative selective advantage (RSA), between the percentage of anti-HIV ribozyme expressing cells after HIV infection and without HIV inoculation was created.

Wild type $CD4^+$ T-cells were mixed with 2–6% autologous LHPLtr or LHPLtrIA transduced $CD4^+$ T-cells. 21 and 28 days after HIV inoculation for all LHPLtr transduced cells a only moderate RSA of approx. 2–2.5 was detected (Fig. 2). For the highly LNGF-R positive subset of cells the RSA was 7.4 ± 1.3 at day 21 and 11.7 ± 4.6 at day 28, i.e. the proportion of $CD4^+$ T-cells with a strong ribozyme expression was more than ten times higher after a selective pressure of four weeks (Fig. 2). For Hut78 cells the RSA was only moderate. A significant RSA could be only observed with highly ribozyme expressing cells (data not shown).

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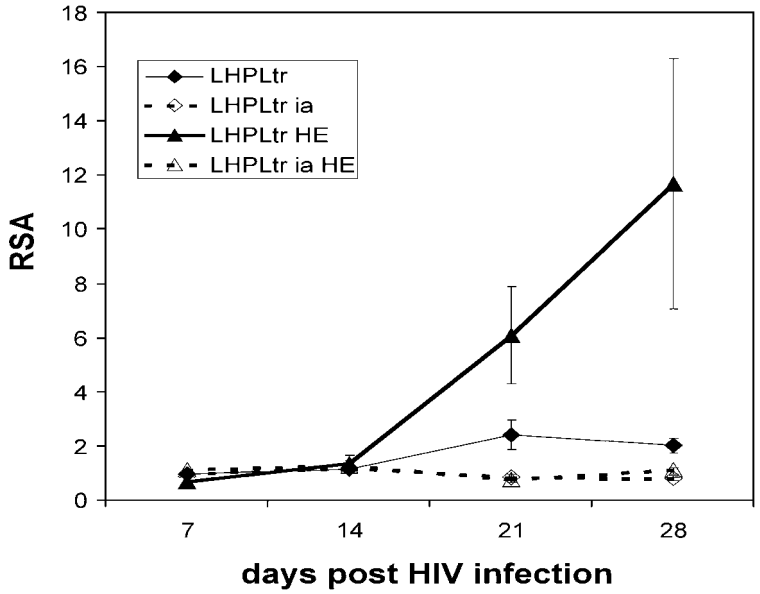

Figure 2. The kinetics of the relative selective advantage (RSA) of LHPLtr or LHPLtrIA expressing $CD4^+$ T-cells 7, 14, 21 and 28 days post HIV infection. The RAS was calculated for all LNGF-R positive cells and for only highly LNGF-R expressing $CD4^+$ T-cells (> Fl-1 channel 200 [HE]) Depicted are the mean ± SE of eight independent experiments.

To elucidate the mechanism of the selective advantage the rate of apoptotic cell death was determined flow cytometrically by 7-AAD staining. Ribozyme or control vector transduced Hut78 were purified and subsequently HIV infected. Table 1 shows the rate of apoptotic cell death 9 days after HIV inoculation. The percentage of apoptotic cells was 16.9 ± 3.5 for LHPLtr expressing and 24.4 ± 0.5 for control vector transduced cells. Since the selective advantage was demonstrated to be dose dependent the rate of inhibition of apoptosis was analyzed separately for cells with a strong LNGF-R expression. 24.1 ± 0.9% of the highly control vector expressing cells versus 9.8 ± 4.7% of the highly LHPLtr expressing cells were apoptotic.

Table 1. Inhibition of Apoptosis by Anti-HIV Ribozymes

	without HIV		with HIV	
	all	HE	all	HE
LHPLtrlA	7,6 +/- 0,9	4,2 +/- 0,4	24,4 +/- 0,4	24,1 +/- 0,9
LHPLtr	7,3 +/- 0,8	4,0 +/- 0,4	17,1 +/- 3,6	9,9 +/- 4.8

The percentage of apoptotic cells among LHPLtr or LHPLtrIA transduced Hut78 cells 9 days after HIV infection or without HIV challenge. The rate of apoptotic cells was determined for all LNGF-R and highly (HE) LNGF-R expressing cells (n = 3, mean ± SE).

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The presented assay system led to the proof of principle for gene therapy with anti-HIV ribozyme expressing $CD4^+$ T-cells. However, even with primary $CD4^+$ T-cells a relevant selective advantage could be observed only in the case of an extremely high level of ribozyme expression, which was almost 10-fold above the average expression level. The very moderate in vitro selective advantage for the whole population of ribozyme expressing cells is in line with the failure to detect a selective advantage in a clinical setting so far. We therefore suggest that a much higher expression level of anti-HIV ribozymes may be required for a clinical application.

To exclude an insufficient inhibition of HIV replication by the ribozyme to be responsible for the limited selective advantage HIV replication was monitored in Hut78 culture supernatants by p24 ELISA. In comparison to earlier published data the inhibition of HIV replication was very effective even with the bulk culture of all ribozyme expressing cells (data not shown). In the first three weeks HIV-1 replication was inhibited by 3 logs in four independent experiments. In the following weeks HIV replication reached the level of the control vector transduced cells. When the same experiments were performed with highly ribozyme expressing Hut78 cells, HIV replication was inhibited completely until the end of the monitoring after seven weeks (data not shown).

In summary, a selective advantage was demonstrated for anti-HIV ribozyme expressing cells. The selective advantage was shown to be dose dependent; only $CD4^+$ T-cells with a strong ribozyme expression substantially benefit from the ribozymes. These results provide evidence that the degree of ribozyme expression achieved by conventional retroviral vectors is not sufficient to provide a RSA. Thus, superior vector systems providing a significantly stronger ribozyme expression are required.

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